The antagonistic regulation of human *MUC4* and *ErbB-2* genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells

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The human transmembrane mucin MUC4 is aberrantly expressed in 75% of pancreatic ductal adenocarcinomas, whereas no expression is found in normal pancreas. Therefore MUC4 appears as a useful biological marker for the diagnosis of ductal adenocarcinomas. Since rat Muc4 was shown to interact with ErbB-2 tyrosine kinase receptor and to either promote cell survival and differentiation or cell proliferation, it is postulated that MUC4 may also participate in pancreatic carcinogenesis. Our aim was to investigate in parallel the role of the Ets factor PEA3 in MUC4 and *ErbB-2* transcriptional regulation in pancreatic cancer cells. Two MUC4-expressing WD (well-differentiated) (CAPAN-1 and -2) and one MUC4-non-expressing poorly differentiated (PANC-1) cell lines were used. The three cell lines express ErbB-2 at different levels. By co-transfection and site-directed mutagenesis, we show that PEA3 is a transactivator of the MUC4 promoter and that the -216 and -2368 PEA3 binding sites of the MUC4 promoter are essential. We also demonstrate that PEA3 acts in synergy with c-Jun and specificity protein 1 to transactivate the proximal region of the *MUC4* promoter and increase MUC4 mRNA levels in WD cells. These results suggest that *MUC4* is a new target gene of the Ets factor PEA3 in pancreatic cancer cells. In contrast, PEA3 represses the transcriptional activity of two fragments of the *ErbB-2* promoter in a dose-dependent manner and decreases the endogenous ErbB-2 mRNA levels in WD cell lines. Thus, PEA3, by its capacity to up-regulate the epithelial marker MUC4 and to down-regulate the *ErbB-2* oncogene, appears as a key regulator of the differentiation/proliferation balance in pancreatic cancer cells.

Key words: carcinogenesis, ErbB-2, MUC4, mucin, PEA3, transcription.

INTRODUCTION

Pancreatic cancer is an especially devastating form of cancer, with only 3% of patients found to survive five years after diagnosis. It constitutes the fifth leading cause of cancer-related death in Western countries. The poor prognosis of this disease is linked to the fact that it affects a rather unaccessible organ and remains asymptomatic until well advanced. Its diagnosis is therefore made at a late stage, where peritoneal dissemination and liver metastasis have already occurred. Moreover, pancreatic cancer is difficult to treat because of its resistance to chemotherapy and radiotherapy. A better understanding of the molecular mechanisms of pancreatic carcinogenesis may lead to the definition of new therapeutic targets and efficient chemotherapeutic interventions. Pathologically, most pancreatic cancers correspond to DAC (ductal adenocarcinoma), which develop from ductal epithelium. Hruban et al. [1] recently proposed a progression model for pancreatic cancer from intraepithelial neoplasia to invasive DAC similar to the adenoma carcinoma sequence described in the colon. In the pancreatic carcinogenetic sequence, K-ras activation and ErbB-2 overexpression are the earliest genetic events [2].

Mucins are high-molecular-mass O-glycoproteins encoded by at least ten genes in humans [3], which exhibit an aberrant pattern of expression in various human malignancies. The common structural feature of all mucins is the presence of tandemly repeated amino acid regions, which serve as docking sites for O-glycans.

Two groups of mucins are now distinguished [3]: (i) secreted mucins including gel-forming (MUC2, MUC5AC, MUC5B and MUC6) and non-gel-forming (MUC7) mucins; (ii) membranebound mucins that are supposed to participate in cell-cell and cellextracellular matrix interactions or in cell signalling [4,5]. Human MUC4 belongs to this last group. It encodes a large transmembrane mucin characterized by the presence of a repetitive domain rich in serine and threonine in its N-terminal region, two EGF (epidermal growth factor)-like domains, a transmembrane domain and a short cytoplasmic tail in its C-terminal part [6]. MUC4 is the human homologue of rat SMC/Muc4, a heterodimeric glycoprotein made of a mucin subunit ASGP-1 tightly bound to a transmembrane subunit ASGP-2. ASGP-2 contains two EGF-like domains in its extracellular part, one of which, EGF1, acts as a ligand for the *ErbB-2/Neu* oncogene [4]. By analogy, MUC4 is supposed to interact with the HER2/ErbB-2 tyrosine kinase receptor.

Several studies have shown that mucin genes are dysregulated in pancreatic cancer. Whereas *MUC4* is not expressed in normal pancreas [7], up to 75% of pancreatic DAC and of pancreatic cancer cell lines show *de novo* expression of *MUC4* at the mRNA level [8]. Recently, Swartz et al. [9] demonstrated a gradual expression of MUC4 at the protein level in pancreatic carcinogenesis with 17% of pancreatic intraepithelial neoplasia type 1 positive for MUC4, whereas in DAC the percentage rises to 89%. The rare expression of *MUC4* in non-neoplastic lesions of the pancreas was also confirmed. According to its high specificity for

Abbreviations used: AP-1, activator protein-1; DAC, ductal adenocarcinoma; EGF, epidermal growth factor; EMSA, electrophoretic mobilty-shift assay; JNK, c-Jun N-terminal kinase; MT, mutated type; PD, poorly differentiated; RT, reverse transcriptase; Sp1, specificity protein 1; WD, well-differentiated; WT, wild-type.

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pancreatic cancer, MUC4 could be used as a biological marker for the histopathological diagnosis of pancreatic adenocarcinoma and for its early detection in the peripheral blood [10]. Moreover, *MUC4* is one of the few genes identified as an attractive target for novel therapeutic strategies in pancreatic cancer [11]. However, at this time, the molecular mechanisms leading to MUC4 dysregulation in pancreatic carcinoma are still poorly understood.

To decipher these mechanisms, we have previously characterized the MUC4 promoter and showed that it contains a GC-rich and TATA-less proximal regulatory region and a distal regulatory region flanked by a TATA box [12]. Several consensus-binding sites for the Ets transcription factors (5'-GGAA/T-3') were found both in the proximal and distal regions of MUC4 promoter. Among those, the proximal putative cis-element (P216) is conserved in the rat Muc4 promoter [13]. The Ets transcription factors have in common an 85-amino-acid helix-turn-helix DNA-binding domain called the ETS domain. Interestingly, the Ets factors have been implicated in a wide range of cellular processes such as differentiation, proliferation and transformation [14]. Recently, Perez et al. [15] have demonstrated that PEA3, a founding member of an Ets protein subfamily, was capable of transactivating the rMuc4 promoter in the mouse mammary epithelial cell line HC11. This effect relies in part on a direct binding of PEA3 to its proximal cis-element P339 (5'-AGGAA-3'), which is not perfectly conserved in the human MUC4 promoter (5'-AGGAG-3'). In light of earlier data on the regulation of ErbB-2 by PEA3 in COS cells [16] and in ovarian cancer cell lines [17], we undertook to investigate in parallel the effects of PEA3 on MUC4 and ErbB-2 regulation in human pancreatic cancer cell lines. As an experimental model, we used two WD (well-differentiated) cell lines, CAPAN-1 and -2, that express high levels of MUC4 mRNA and a PD (poorly differentiated) cell line that does not express MUC4, PANC-1 [12]. The three cell lines express ErbB-2 protein at different levels. The aim of the present study was to demonstrate whether in pancreatic cancer cell lines, PEA3 co-regulates MUC4 and *ErbB-2* that encode two membrane-bound protein partners.

MATERIALS AND METHODS

Cell cultures

Pancreatic cancer cell lines CAPAN-1 and -2 were purchased from A.T.C.C., and PANC-1 from ECACC (European Collection of Animal Cell Cultures). The cells were cultured as described previously [12].

Transient transfections

Cells were passed at 0.5×10^6 cells/well the day before being transfected by pGL3 basic vector (Promega, Charbonnières, France) containing a deletion mutant of the MUC4 promoter upstream of the luciferase gene [12] using Effectene reagent as described previously [18] or by a pXP2 vector containing a deletion fragment of ErbB-2 promoter [19] upstream of the luciferase gene (gift from Dr R. Winkler, Université de Liège, Belgium). Total cell extracts were prepared after a 48 h incubation at 37 °C using 1× RLB (reporter lysis buffer) buffer according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). The luciferase activity and the protein content of the cell extract were measured as described previously [12]. Results were first expressed as luciferase activity/ μ g of protein. To take into account the transfection efficiency, the corrected results were obtained by dividing the luciferase activity/ μ g protein by those of the positive control plasmid (pGL3-control vector). Final results are expressed as fold of induction of the pGL3-control vector. In co-transfection

studies, cells were transfected with pGL3 MUC4 promoter deletion mutants in the presence of an expression vector encoding either mPEA3 (pSV-HA-mPEA3, gift from Dr J. L. Baert, Institut Pasteur de Lille, France), ER81 (pSV-ER81), ERM (pSV-ERM, both were gifts from Professor Y. de Launoit, Université Libre de Bruxelles, Belgium), Sp1 (specificity protein 1; pCMV-Sp1, a gift from Dr J. M. Horowitz, Raleigh, U.S.A.) or c-Jun (pRSV-c-Jun). Results were expressed as fold induction relative to the co-transfection performed in the presence of the corresponding empty expression vectors. Each combination of plasmids was assayed in triplicate in three separate experiments. Data were analysed by Student's t test with differences t 0.05 considered significant.

Site-directed mutagenesis

The P216 PEA3-binding site was mutated from AGGAA to AAAAA in the -219/-1 deletion mutant using the QuikChange Site-Directed Mutagenesis kit (Stratagene, Saint Quentin en Yvelines, France) according to the manufacturer's instructions. The mutated plasmid DNA was purified and sequenced on both strands before being used in cell transfection experiments.

Nuclear extract preparation and Western blotting

Nuclear and cytoplasmic extracts from cell lines of interest were prepared as described previously [20] and kept at -80 °C before use. Protein content was determined using the bicinchoninic acid method. Nuclear or cytoplasmic proteins (25 μ g) were separated by SDS/10% PAGE, followed by an electrotransfer on to a $0.2 \mu m$ nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were first incubated with specific primary antibodies [anti-PEA3 mouse monoclonal (sc-113), antic-Jun rabbit polyclonal (sc-44) and anti-Sp1 goat polyclonal antibody (sc-59) all from Santa Cruz Biotechnology (Tebu, France); anti-ErbB-2 mouse monoclonal antibody (NCL-CB11) from Novo Castra (Tebu, France)] before a second incubation with a peroxidase-conjugated secondary antibody (PEA3, c-Jun and erbB-2) or alkaline phosphatase conjugated IgG (Sp1). Immunoreactive bands were visualized using the chemiluminescence Super Signal system (PEA3, c-Jun and ErbB-2) and Hyperfilm ECL® (Amersham Biosciences, Little Chalfont, Bucks., U.K.) or (Sp1) Nitro Blue Tetrazolium Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, Cergy-Pontoise, France). The autoradiograms were scanned and a densitometry analysis was performed using the Clara Vision Gel Smart-Gel Analysis software. Values were expressed in arbitrary units.

EMSAs (electrophoretic mobility-shift assays)

Binding studies were performed with double-stranded oligonucleotides containing the P216 PEA3-binding site of the MUC4 proximal promoter (sense oligonucleotide: 5'-CGGTGTTTTCTTCCTGGTGG-3'). The oligonucleotides were annealed before being phosphorylated at the 5'-end with $[\gamma^{-3^2}P]$ ATP (Amersham Biosciences, Saclay, France) and T4 polynucleotide kinase. The binding reaction was performed by preincubating 9 μ g of nuclear extract in 10 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 12.5 % (v/v) glycerol and 2 μ g of poly(dI-dC) · (dI-dC) in a final volume of 20 μ l for 10 min at room temperature (25 °C). Then the radiolabelled probe (90 000 c.p.m.) was added to the reaction mixture and incubated for an additional 30 min at room temperature as described previously [21]. Competition experiments using a 100-fold molar excess of WT (wild-type) or mutated (sense oligonucleotide:

5'-CGGTGTTTTCTTTGGTGGTGG-3') unlabelled probe were performed as described before [21]. Supershift experiments were performed by adding 2 μ l of an anti-PEA3 or anti-ErbB-2 antibody to the reaction mixture together with the radiolabelled probe [21]. The reaction was stopped by adding 2 μ l of $10\times$ loading buffer. Samples were run on a 6% non-denaturating polyacrylamide gel (200 V for 3 h at 4°C) with $0.5\times$ TBE (44.5 mM Tris, pH 8.3, 44.5 mM boric acid, 1.25 mM EDTA) buffer. Gels were dried and autoradiographed. The PEA3 fusion protein was prepared using the pSV-HA-mPEA3 expression vector and the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. The binding reaction was performed with 5 μ l of the PEA3 fusion protein preparation using the experimental method as described by Perez et al. [15].

RT (reverse transcriptase)-PCR analysis

Total RNAs from pancreatic cancer cells were prepared using the RNeasy mini-kit (Qiagen, Courtaboeuf, France) as described earlier [12]. cDNA was synthesized from 1.5 μ g RNA using the Advantage-RT for PCR kit (BD Bioscience, Saint Quentin en Yvelines, France). To evaluate MUC4 expression, PCR was performed on 2 μ l of cDNA using previously described primers [12]. To evaluate PEA3 expression, a specific pair of primers was designed (E1Af: 5'-ACCATGGCGAGCAGTGCCTT-3' and E1Ar: 5'-TCCGACAGCTGGTGTTGGTA-3'), which amplifies a 288 bp fragment outside the ETS domain that starts in exon 6 and ends in exon 8 of the human gene. PCR was performed in a final reaction volume of 50 μ l containing 5 μ l of cDNA, 2 μ l of Ampli Taq (Roche, Meylan, France), 10 pmol of each primer and 1.5 mM MgCl₂ using the following programme: denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min; primer annealing at 57 °C for 1 min and elongation at 72 °C for 1 min. Final elongation was conducted at 72 °C for 5 min. To check the specificity of the primers, the 300 bp band was gelpurified before being sequenced using the ABI PRISM 377 XL automatic sequencer and the ABI PRISM Ampli Taq FS Big Dye Terminator cycle sequencing ready reactions kit (Applied Biosystems, Courtaboeuf, France). The nucleotide sequence was analysed by the EMBL FASTA Server [22] showing a 100 % identity with the sequence of PEA3/E1AF mRNA (GenBank® accession no. D12762). To evaluate ErbB-2 expression, we used the following pair of primers (NAU2126: 5'-CGCGAGCTCGG-AGGACGAGTGTGTGGG-3' and NAU2127: 5'-CGCGGTAC-CCAGAATGCCAACCACCGC-3'), which amplifies a 485 bp fragment that showed a 100 % identity with the human c-erb-B2 mRNA (GenBank® accession no. X03363). The PCR programme was as follows: denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s; primer annealing at 60°C for 30 s and elongation at 72°C for 30 s. Final elongation was conducted at 72 °C for 5 min. 28S or β -actin was used as internal control.

Immunohistochemistry

Cells were passed at 1.5×10^6 cells/75 cm² flask 48 h before being transfected with 4 μg of vector as described before. After 48 h, cells were trypsinized, centrifuged and washed once with $1 \times$ PBS. The pellet was fixed in 4 % (w/v) formaldehyde, embedded in paraffin and 3 μm sections were prepared. Immunohistochemistry was performed on serial sections of the same blocks using an automatic immunostainer (ES; Ventana Medical System, Strasbourg, France) as described previously [23]. The monoclonal anti-MUC4 antibody, which recognized the tandem repeat region of the protein [9], was used at a 1/10000 dilution.

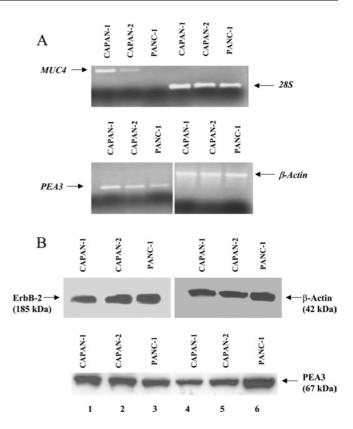


Figure 1 (A) Analysis of the expression of MUC4 and PEA3 mRNAs in pancreatic tumour cells by RT-PCR and (B) expression of ErbB-2 and PEA3 proteins by Western blotting in pancreatic cancer cells

(A) Cells were cultured in 75 cm² flasks to 80 % confluence. RT–PCR was performed using specific primer pairs as described in the Materials and methods section. 28 S or β -actin was used as internal control. PCR products were separated on 1.5 % agarose gel containing ethidium bromide in 1× TBE buffer. The size of the PCR products were as follows: MUC4, 596 bp; 28 S, 231 bp; β -actin, 661 bp. (B) Cells were cultured in 75 cm² plates at 100 % confluence before being scrapped in PBS buffer. Cytosolic and nuclear extracts were prepared as described in the Material and methods section and then separated on SDS/10 % PAGE (25 μ g). Proteins were electrotransferred on to nitrocellulose and the blot was probed with a specific anti-human PEA3 antibody. For ErbB-2 analysis, total cellular extracts were used (see the Materials and methods section) and separated by SDS/8 % PAGE (20 μ g).

RESULTS

Expression of MUC4, ErbB-2 and PEA3 in pancreatic cancer cells

Expression of the different partners studied in the present study was evaluated by RT-PCR or Western blotting. As already shown in [12], MUC4 mRNA was found only in the WD cell lines CAPAN-1 and -2 (Figure 1A). PEA3 expression was first evaluated by RT-PCR using a specific pair of primers designed to amplify a 5'-region of human PEA3 cDNA outside the conserved ETS domain. The results showed that the three cell lines express PEA3 mRNA (Figure 1A), at an apparently lower level in PANC-1 cells. To look for statistically significant differences, a PEA3/ β actin ratio was calculated on two independent experiments and a t test was performed, showing that CAPAN-1 [mean ratio (S.D.), 0.865 (0.007)], CAPAN-2 [0.945 (0.106)] and PANC-1 cells [0.616 (0.220)] express similar levels of PEA3. We also examined PEA3 expression at the protein level by Western blotting using nuclear (lanes 1-3) and cytosolic (lanes 4-6) extracts prepared from the three cell lines (Figure 1B). PEA3 is found at higher levels in CAPAN-1 (lane 1) and CAPAN-2 (lane 2) nuclear extracts when compared with PANC-1 (lane 3). In contrast,

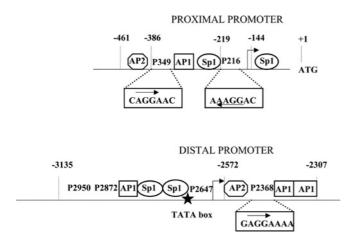


Figure 2 Schematic representation of the proximal and distal regulatory regions of *MUC4* promoters

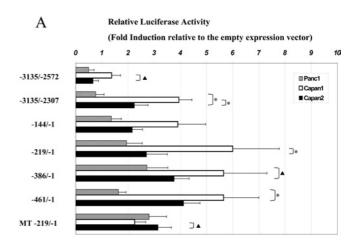
The numbering refers to the A of the first ATG, and the main *cis*-elements for Sp1, AP-1, AP-2 and PEA3 (Pxxx) are shown. The bent arrows represent the transcriptional start site locations. The nucleotide sequence and the orientation of the PEA3 *cis*-elements are boxed.

PANC-1 cytosolic extracts (lane 6) contain higher amounts of PEA3 than CAPAN-1 (lane 4) or CAPAN-2 (lane 5). Finally, we controlled the levels of ErbB-2 protein expressed by the three cell lines by Western blotting (Figure 1B). The densitometry analysis of the bands showed that CAPAN-1 cells expressed the lowest levels of ErbB-2 (P < 0.05 versus CAPAN-2, n = 2), whereas CAPAN-2 and PANC-1 expressed similar levels [mean $ErbB-2/\beta$ -actin ratio (S.D.), 0.89 (0.014) for CAPAN-1, 1.50 (0.009) for CAPAN-2 and 1.11 (0.24) for PANC-1].

PEA3 selectively transactivates the promoter of MUC4

The promoter of MUC4 contains several canonical Ets-binding sites (5'-AGGAA-3') located in the proximal transcriptional unit (P216 and P349) upstream of the transcription start site (Figure 2, upper panel) and in the distal transcriptional unit upstream (P2950, P2872 and P2647) or downstream (P2368) of the transcription start site (Figure 2, lower panel). To study the effects of the Ets protein PEA3 on the MUC4 promoter, we co-transfected a panel of deletion mutants covering both promoters of MUC4 [12] in the presence of an expression vector encoding full-length PEA3. In CAPAN-1 cells (Figure 3A, white bars), PEA3 strongly transactivates the proximal promoter especially the -219/-1 region containing the PEA3 site P216. A stronger effect is not seen with longer fragments outlining the importance of this site. Mutagenesis of P216 (5'-AGGAA-3' to 5'-AAAAA-3') led to a 60 % reduction of the PEA3 transactivating effect on the -219/-1region (Figure 3A, MT -219/-1, where MT stands for mutated type). When the luciferase activities of WT (WT -219/-1) and mutant (MT - 219/-1) constructs were compared in the absence of PEA3, we observed a 70 % reduction of activity for the mutated form (Figure 3B). This indicates that the PEA3 P216 site acts as an important positive regulatory element required for MUC4 promoter activity in CAPAN-1 cells.

In CAPAN-2 cells (Figure 3A, black bars), which express lower levels of MUC4 mRNA (see Figure 1A), the transactivating effect of PEA3 on the -144/-1 and -219/-1 regions was 50% less intense than in CAPAN-1 cells. Interestingly, the response to PEA3 in these cells increased as longer fragments of the *MUC4* promoter were transfected. These results suggest that positive elements for PEA3 regulation are present in the -461/-219



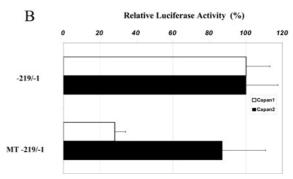


Figure 3 PEA3 transactivates the MUC4 promoter in a cell-specific manner in pancreatic tumour cell lines

(A) The MUC4-pGL3 deletion mutants were transiently transfected in CAPAN-1, -2 and PANC-1 cells in the presence of 0.2 μg of pSV-mPEA3 expression vector or empty pSV. The MT-219/-1 fragment represents the -219/-1 region in which the PEA3 cis-element P216 was mutated. Results are expressed as fold induction of luciferase activity relative to the empty expression vector. Values are means for three independent experiments, where co-transfections were run in triplicate. The error bar represents the S.D. from the mean. \blacktriangle , $P<0.05; ^*$, P<0.01. (B) Effect of mutation of the P216 site on the luciferase activity driven by the -219/-1 region in CAPAN-1 and -2 cells. The luciferase activity of the WT region (WT-219/-1) was arbitrarily set at 100 %. Results are expressed as means \pm S.D. for three independent experiments

region of the promoter. Accordingly, the mutation of the conserved PEA3 site P216 did not significantly affect the transcriptional activity of the -219/-1 region in CAPAN-2 cells (Figure 3B). PEA3 also induces the luciferase activity driven by the -3135/-2307 region of the MUC4 promoter in CAPAN-1 (4-fold) and CAPAN-2 (2-fold) cells (Figure 3A), whereas it does not modify the activity of the -3135/-2572 region. The -3135/-2307 region contains one additional Ets-binding site (P2368) and two closely linked AP-1 (activator protein-1) cis-elements compared with the -3135/-2572 region. Thus these sites are probably crucial for PEA3 transactivating effect in those two cell lines.

Finally, the weakest effects of PEA3 were observed in the MUC4-non-expressing cell line PANC-1 (Figure 3A, hatched bars) in which the transactivating effect of PEA3 remained < 2-fold except for the -386/-1 region of the proximal promoter, where it reached 2.7 (2.7 \pm 0.8). Nevertheless, this value was significantly lower than those obtained in CAPAN-2 cells (3.8 \pm 0.57, P < 0.05, Student's t test).

To check whether PEA3 transactivating effect on the *MUC4* promoter is shared by other members of its subfamily, co-transfection studies were performed using expression vectors encoding

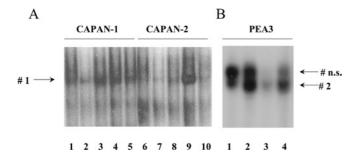


Figure 4 DNA binding of PEA3 to the P216 cis-element of MUC4 proximal promoter

(A) Nuclear extracts from CAPAN-1 and -2 cells were used in EMSA experiments as described in the Material and methods section. A specific retarded complex (arrow #1) was seen both in CAPAN-1 (lane 1) and CAPAN-2 (lane 6) cells. Specificity was assessed by the addition of a 100-fold excess of an unlabelled WT probe (lanes 2 and 6) or mutated probe (lanes 3 and 7). Supershift experiments were performed by adding 2 μ l of an anti-PEA3 antibody (lanes 4 and 9) or an anti-ErbB-2 antibody (lanes 5 and 10). (B) The DNA-binding activity of a full-length mouse PEA3 protein was evaluated by EMSA. Of the transcription—translation reaction, 5 μ l was performed without plasmid DNA (control reaction, lane 1) or 5 μ l of mPEA3 protein (lanes 2 and 3) was incubated with a radiolabelled T76 probe. A non-specific DNA—protein complex (#n.s.) is observed both in lanes 1 and 2. The specific complex (#2) corresponds to the high mobility band that disappeared on addition of an excess of unlabelled probe (lane 3) but decreased only weakly on addition of an excess of the mutated probe (lane 4).

ER81 or ERM both in CAPAN-1 and -2 cells. Three fragments of the MUC4 promoter corresponding to either the -3135/-2307, -219/-1 or -461/-1 region were chosen. In CAPAN-1 cells, ER81 and ERM did not transactivate the proximal or the distal regions of MUC4 promoter thus demonstrating an effect restricted to PEA3. In contrast, in CAPAN-2 cells ER81 and ERM are at least as potent transactivators as PEA3 on the three regions of the MUC4 promoter (results not shown).

We then checked whether PEA3 could bind to the P216 binding site by gel-retardation analysis. As shown in Figure 4(A), one retarded complex (lanes 1 and 6, arrow #1) was obtained when incubating CAPAN-1 or -2 nuclear extracts with the T₇₆ radiolabelled probe containing the P216 site (5'-AGGAA-3'). Competition experiments performed with a 100-fold excess of unlabelled T₇₆ oligonucleotide led to a substantial decrease of the shifted band (lanes 2 and 7), whereas an oligonucleotide with a mutated PEA3-binding site (T_{76MT}: 5'-ACCAA-3') did not modify the binding (lanes 3 and 8). Therefore the data indicate that an Ets protein is involved in the formation of the retarded complex. Surprisingly, no supershift was observed when an anti-PEA3 antibody was added in the binding reaction (lanes 4 and 9), instead a strong increase in the retarded band was noticed, being more pronounced with CAPAN-2 nuclear extracts (compare lane 9 to lane 6, and lane 4 to lane 1). The addition of an irrelevant antibody (anti-ErbB-2) to the medium did not modify the retarded band formation (lanes 5 and 10). To show that PEA3 binds directly to the P216 site, we produced a fusion protein encoding a HA-mPEA3 fusion protein and checked its ability to bind to the T_{76} probe. As shown in Figure 4(B), the high mobility band (arrow #2, lane 2) represents a specific DNA-protein complex. The upper band is not specific as it was also found in the control reaction containing no added DNA plasmid (lane 1, n.s.). The specificity of complex #2 was confirmed by performing unlabelled competition with a 100-fold excess of T₇₆ that led to a total loss of the shifted band (lane 3). In contrast, when the $T_{76\text{MT}}$ oligonucleotide with a mutated PEA3-binding site was used, the intensity of the shifted band decreased very slightly (lane 4). This shows that PEA3 can bind specifically to the P216 site in the MUC4 promoter.

c-Jun and Sp1 transactivating effects on the promoter of MUC4

PEA3 is often considered as a weak transcription factor, which needs to co-operate with partners such as Spl and c-Jun to transactivate efficiently its target genes [14]. Moreover, both the proximal and distal promoters of MUC4 have several consensus putative cis-elements for AP-1 and Sp1 transcription factors (see Figure 2). To show whether Sp1 and/or AP-1 co-operate with PEA3 to activate MUC4 transcription, we co-transfected the -461/-1 and -219/-1 regions of the proximal promoter and the -3135/-2307 region of the distal promoter with expression vectors encoding PEA3, Sp1 or c-Jun respectively (Figure 5). The -461/-1 region, which is the most responsive to PEA3 in CAPAN-2 cells (see Figure 3A), is also stimulated by Sp1 (3-fold induction; Figure 5A, black bars) or c-Jun (Figure 5A, black bars). Interestingly, co-expression of Sp1 greatly increases the transactivating effect of PEA3 on the -461/-1 region in CAPAN-2 cells (12-fold; P < 0.001 versus PEA3 alone). This is not observed on the shorter -219/-1 region (Figure 5B) suggesting that the upstream Sp1 and the P349 PEA3 cis-elements (Figure 2) are required for this synergistic effect. An additive effect is seen between c-jun and PEA3 on the -461/-1 region (P < 0.05) becoming synergistic on the shorter -219/-1 in CAPAN-2 cells (10-fold induction, P < 0.01). Since this last region does not contain a canonical AP-1 cis-element, these data suggest that c-Jun acts indirectly probably by increasing PEA3 binding to the P216 binding site as proposed previously [24]. In contrast, Sp1 does not increase the transactivating effect of PEA3 on the -461/-1 or the -219/-1 regions in CAPAN-1 cells (Figures 5A and 5B, white bars). A weak synergistic effect is noticed between c-Jun and PEA3 on the -219/-1 region in these cells (9-fold induction). In PANC-1 cells (hatched bars), the weak transactivating effect of PEA3 on the two proximal regions of the MUC4 promoter is not modified by the co-expression of either Sp1 or c-Jun. Taken together, these results indicate that strong synergistic effects between PEA3/Sp1 or PEA3/c-jun occur on the MUC4 proximal promoter in CAPAN-2 cells.

Concerning the distal promoter of MUC4, a 4-fold activation by c-Jun is obtained on the -3135/-2307 region in CAPAN-1 cells (Figure 5C) making this transcription factor as potent as PEA3. Interestingly, c-Jun and PEA3 act in synergy to transactivate this region both in CAPAN-1 (17-fold induction, P < 0.001) and CAPAN-2 cells (10-fold, P < 0.05). This effect is not seen on the -3135/-2572 region (results not shown). Sp1, alone or with PEA3, does not modify the luciferase activity driven by the -3135/-2307 region in either cell line (Figure 5C). Taken together, these results outline the important role of the P2368 PEA3 site and of the two adjacent AP-1 cis-elements (see Figure 2) to convey synergistic activation of the MUC4 distal promoter in both WD cell lines. c-Jun could either act directly by binding to its cis-elements and/or indirectly by increasing the binding of PEA3 to its cognate element.

PEA3 and its partners regulate MUC4 mRNA levels in pancreatic cancer cells

The response of the -3135/-2307 region to PEA3 and c-Jun in CAPAN-1 and -2 cells, and those of the -461/-1 and -219/-1 regions to PEA3 and Sp1 and PEA3 and c-Jun in CAPAN-2 cells respectively prompted us to evaluate whether these two combinations of transcription factors also affected the level of endogenous MUC4 mRNA in WD pancreatic cancer cells. To this end, we transiently co-transfected these cells as described before and isolated total RNA 48 h later. RT–PCR (Figure 6A) and the densitometry analysis of the bands (Figure 6B) show that: (i) PEA3, Sp1 and c-jun alone did not modify MUC4 mRNA

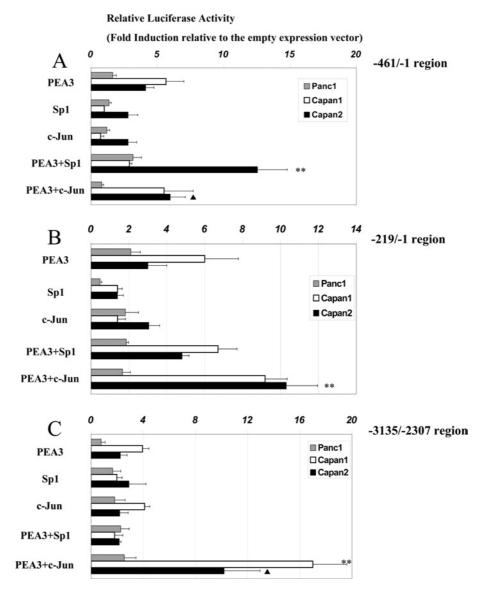


Figure 5 PEA3 acts in synergy with c-Jun or Sp1 to transactivate different regions of MUC4 promoter

PEA3, Sp1 and c-Jun were transiently co-transfected alone or in combination in pancreatic tumour cells with fragments encoding the -461/-1 (**A**), -219/-1 (**B**) or the -3135/-2307 (**C**) regions of MUC4 promoters. Results are expressed as fold induction relative to the empty expression vector. Results are means for three independent experiments, where co-transfections were run in triplicate. \triangle , P < 0.05; **, P < 0.001 versus PEA3 alone.

levels in both WD cell lines; (ii) PEA3 + Sp1 strongly increased MUC4 expression in CAPAN-1 cells by 221 %; (iii) PEA3 + c-Jun increased MUC4 expression by 160 % both in CAPAN-1 and -2 cells. None of these combinations was capable of inducing the expression of *MUC4* in the PD PANC-1 cells (results not shown). The absence of the induction of endogenous MUC4 expression with 0.2 μ g of PEA3 expression vector was unexpected and, thus, considered to be dose-dependent. Therefore higher amounts of the expression vector (0.5, 1 or $2 \mu g$) were assayed and the level of MUC4 mRNA was measured as described previously. The results confirmed the hypothesis since in CAPAN-1 cells a high activation (5.7-fold) was obtained with 1 μ g of PEA3 expression vector. In CAPAN-2 cells, an induction was also observed but remained weaker with a maximal value of 2.1-fold with 1 μ g of expression vector. In conclusion, our results demonstrate that PEA3 alone, in a dose-dependent manner or in association with c-Jun and/or Sp1, is a potent activator of MUC4 expression in the WD CAPAN-1 and -2 cells.

PEA3 regulates MUC4 apomucin expression in pancreatic cancer cells

Having shown that PEA3 transactivates the *MUC4* promoter in CAPAN-1 and -2 cells and induces MUC4 mRNA levels in these cell lines, we chose to evaluate its effects at the protein level. To this end, cells were transfected with either an empty expression vector or with pSV-HA-mPEA3, and then processed for immunostaining with a specific anti-MUC4 antibody. In CAPAN-1 mock cells, 38 % of the cells were positively labelled with anti-MUC4 antibody (Figure 7A). The staining was restricted to the cytoplasm. Transfection with PEA3 expression vector led to an increase in the percentage of positive cells to 60% accompanied by an increase in the staining intensity (Figure 7C). In CAPAN-2 mock cells, 53 % of the cells were positively labelled with the anti-MUC4 antibody (Figure 7B), a percentage that was not significantly modified to 56% in the PEA3-transfected cells (Figure 7D). The immunostaining intensity was weaker in

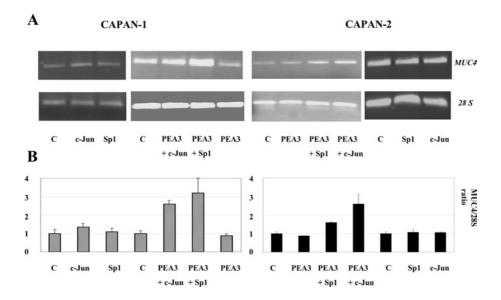


Figure 6 (A) Effects of c-Jun, Sp1 or PEA3 alone or in combination on the expression of endogenous MUC4, (B) representation of the level of MUC4 mRNA expressed by the cell lines

(A) Cells were cultured in 40 mm dishes at 40 % confluence before being transfected with 0.2 μg of expression vectors encoding PEA3, Sp1 and/or c-Jun or the empty expression vector (C, control). Analysis of MUC4 mRNA was assessed by RT–PCR as described in the Materials and methods section. 28S was used as an internal control. (B) Results are expressed as the MUC4/28S ratio after densitometry analysis of the bands. The ratio obtained with the empty expression vector was set at 1. The data shown are means ± range for two independent experiments.

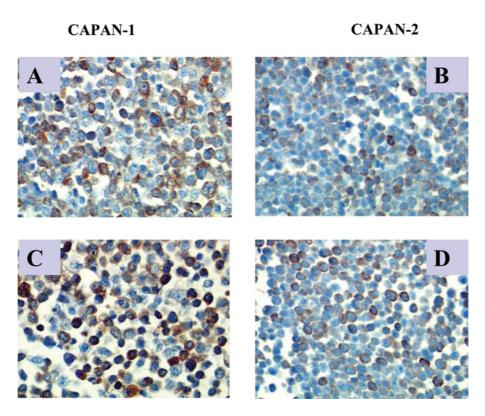


Figure 7 Effect of PEA3 on MUC4 apomucin expression in CAPAN-1 and -2 cells

Immunohistochemistry was performed as described in the Materials and methods section. CAPAN-1 cells transfected by 4 μ g of a control empty vector (**A**) or PEA3 expression vector (**C**). CAPAN-2 cells transfected by 4 μ g of a control empty vector (**B**) or PEA3 expression vector (**D**). Magnification \times 200.

CAPAN-2 cells when compared with that in CAPAN-1 cells. In conclusion, our results showed that PEA3 is also an activator of MUC4 apomucin expression in CAPAN-1 cells.

PEA3 and its partners are expressed in pancreatic cancer cells

Having demonstrated a strong correlation between the transactivating effect of PEA3 on MUC4 promoter activity (Figure 3A)

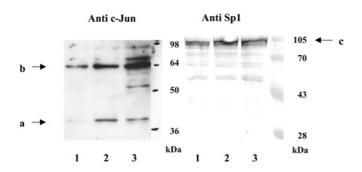


Figure 8 Analysis of the expression of c-Jun and Sp1 in nuclear extracts of PANC-1 (lane 1), CAPAN-2 (lane 2) and CAPAN-1 (lane 3) cells by Western hlot

Nuclear proteins ($20~\mu g$) were separated on SDS/10% PAGE before being transferred on to nitrocellulose. The blot was stained using specific anti-c-Jun or anti-Sp1 antibody. Migration of the molecular-mass standard is shown on the right-hand side. The arrows point at (a) the monomeric c-Jun (approx. 40 kDa); (b) dimeric c-Jun; (c) Sp1 protein (\approx 105 kDa).

and the level of MUC4 mRNA expressed by the three pancreatic cancer cell lines (Figure 1), we undertook to determine the level of expression of PEA3 and its partners in these cells. As previously shown, PEA3 is found at higher levels in the nuclear extracts of the WD cell lines than in those of the PD PANC-1 cells. These results suggest a cytosolic sequestration of PEA3 in PANC-1 cells that may prevent it exerting its transcriptional effects. Moreover, since the different levels of MUC4 expression in the three cell lines may be due to a different pattern of expression of c-Jun, and/or Sp1 that co-operate with PEA3, we determined their levels of expression in the same nuclear extracts by Western blotting (Figure 8). c-Jun is mainly found under the dimeric form (band b) in the three cell lines. Interestingly, the intensity of the band corresponding to the dimeric form of c-Jun is much stronger in CAPAN-1 (lane 3) and CAPAN-2 (lane 2) cells when compared with that in PANC-1 (lane 1) suggesting that c-Jun is engaged in active transcriptional complexes in MUC4-positive cell lines. The amount of Sp1 (band c) is similar in the three cell lines.

PEA3 down-regulates the expression of ErbB-2 in pancreatic cancer cells

Since MUC4 is the ligand of ErbB-2, we checked whether they were both positively co-regulated by PEA3. To this aim, co-transfection experiments were performed using two proximal fragments of the human ErbB-2 promoter, i.e. the p255 and the p537 fragments [19]. The p255 fragment contains a conserved consensus PEA3-binding site (5'-AGGAA-3') near the TATA box and several core elements (5'-GGAA-3') for Ets proteins; the p537 fragment contains one more PEA3 putative binding site near GATA-1 and AP-1-binding sites [19]. The transfection experiments showed that in CAPAN-1, -2 and PANC-1 cells, PEA3 repressed the transcriptional activity of the two fragments of the ErbB-2 promoter in a similar way (Figure 9A). This suggests that the proximal PEA3 consensus-binding site in the p255 fragment is sufficient to mediate PEA3 inhibitory effect on the ErbB-2 promoter. Moreover, the down-regulation of the ErbB-2 promoter by PEA3 is dose-dependent, as increasing amounts of PEA3 repress the promoter activity of the p255 region up to 90 % in the three cell lines. In conclusion, these results indicate that in contrast with what was observed on the MUC4 promoter, PEA3 is a potent transrepressor of the human *ErbB-2* promoter in pancreatic cancer cell lines.

The inhibition of *ErbB-2* expression by PEA3 was confirmed at the mRNA level. In CAPAN-1 cells and, to a lesser extent in

CAPAN-2 cells, endogenous ErbB-2 mRNA levels substantially decreased when PEA3 was overexpressed in the cells (Figure 9B). The maximum decrease was 75–80% in CAPAN-1 cells (for 1 μ g of expression vector) and 50% in CAPAN-2 cells (for 2 μ g of expression vector, Figure 9C). Thus expression level in cells correlated well with regulatory data at the promoter level. When one compared the levels of expression of PEA3, MUC4 and ErbB-2 (Figure 1) in the three cell lines, it came to our attention that the WD CAPAN-1 cells expressed both high levels of PEA3 and MUC4 and the lowest levels of ErbB-2. In contrast, the PD PANC-1 cells that contained low levels of active PEA3, express ErbB-2 but no MUC4.

DISCUSSION

The aim of the present study was to better understand the mechanisms leading to MUC4 aberrant expression in pancreatic cancer cell lines, and especially to characterize further the transcription factors and the cis-regulatory elements involved in the control of MUC4 expression. In this study, we focused our attention on the Ets transcription factor PEA3 for two reasons. First, both the distal and proximal promoters of MUC4 contain numerous binding sites for Ets transcription factors, among those the P216 is conserved in human [12] and rat [13] promoters. Secondly, PEA3 is a key transcriptional regulator of the proto-oncogene ErbB-2 [16,17]. Moreover, the fact that ratMuc4 was shown to be a ligand of the rat homologue of ErbB-2 (p185neu) [4] suggests a link between MUC4, PEA3 and ErbB-2.

Our results demonstrate that PEA3 acts as a potent transactivator of both regions of the *MUC4* promoter in the *MUC4*-expressing cell lines and that its transactivating effect is correlated to the level of MUC4 mRNA expressed by these cells. We also showed by EMSA experiments that PEA3 binds to the P216 PEA3 element of the *MUC4* promoter and consequently acts directly. The fact that the interaction between PEA3 and its *cis*-element is stronger in the presence of an anti-PEA3 antibody could rely on a relief of PEA3 DNA-binding autoinhibition by preincubation with the antibody as described previously [25]. These results are in agreement with a recent research showing that PEA3 transactivates the proximal promoter of rat *Muc4* in mammary cancer cells [15]. Thus PEA3 appears as an important factor that could up-regulate *MUC4* expression in tumour cells.

Chotteau-Lelievre et al. [26] previously showed using in situ hybridization that in adult mice PEA3 expression is restricted to the brain, skeletal muscles and colon, whereas during development PEA3 is widely expressed. An important point was therefore to check PEA3 expression in pancreatic cancer cells to document its biological role in the control of MUC4 expression. In the present study, we show for the first time that PEA3 is expressed in pancreatic cancer cells, as recently shown in human breast [16] and colon cancer cell lines [27]. Moreover, PEA3 is present at high levels in the nuclear compartment of MUC4-positive WD cell lines allowing it to exert its transactivating effect. In the MUC4negative PD PANC-1 cells, the level of PEA3 mRNA is similar to those observed in the MUC4-positive cells. However, in PANC-1 cells, PEA3 protein is mostly found in the cytosol compartment and did not transactivate the MUC4 promoter. Thus it may be hypothesized that in PANC-1 cells the transduction pathway(s) involved in PEA3 activation, translocation and binding to DNA is (are) altered, preventing it from exerting its transactivating effect or that the MUC4 locus is inactivated by epigenetic mechanisms [28]. Bojovic and Hassell [25] suggested that PEA3 is found in mammalian cells in a dynamic equilibrium between two states: one able and the other unable to bind to DNA. The transition between the active and inactive states depends on

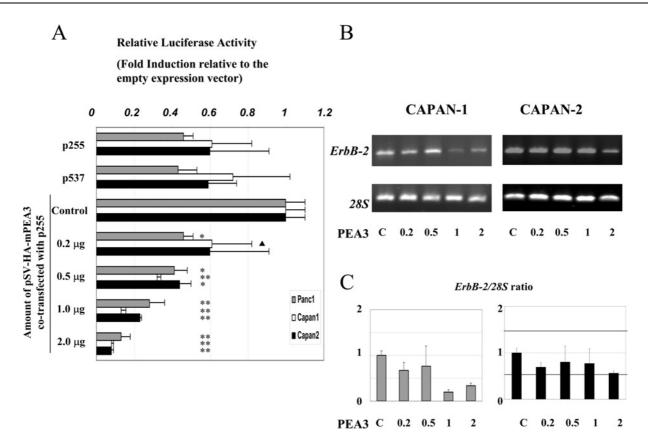
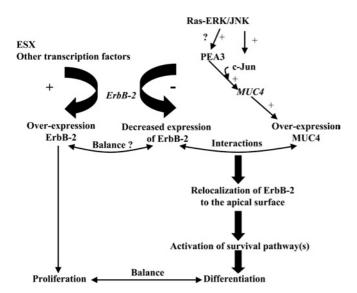


Figure 9 (A) Effects of increasing amounts of PEA3 expression vector on the promoter activity and the expression of the human *ErbB-2* promoter, (B) effects of increasing amounts of PEA3 expression vector on the expression of endogenous *ErbB-2* and (C) representation of the level of ErbB-2 mRNA expressed

(A) Expression vector $(0.2-2~\mu g)$ was transiently co-transfected with deletion fragments of the *ErbB-2* promoter in CAPAN-1, -2 and PANC-1 cells. Results are expressed as in Figure 3(A). \blacktriangle , P < 0.05; *, P < 0.01 and **, P < 0.05 or with empty expression vector. Analysis of ErbB-2 mRNA was assessed by RT–PCR as described in the Materials and methods section. *28S* was used as an internal control. (**C**) Results are expressed as described in Figure 6(B) and are means \pm range for two independent experiments.

post-translational modifications such as phosphorylation [29] and/or on interactions with protein partners. Altogether, the differences seen in the transactivating effect and in the intracellular distribution of PEA3 between MUC4-expressing and nonexpressing cell lines suggest the involvement of PEA3, and possibly of other Ets proteins, in the regulation of MUC4 transcription in pancreatic tumours. The PEA3-mediated increase in MUC4 endogenous expression in CAPAN-1 and -2 cells was dose-dependent and became efficient at high doses of PEA3. This is in agreement with the fact that PEA3 is considered as a weak transactivator that often co-operates with other transcription factors to regulate the expression of target genes [30]. Among the already known partners of PEA3, we focused our attention on Sp1 and c-Jun transcription factors. c-Jun is a major member of the AP-1 family, which regulates a variety of cellular processes such as proliferation, differentiation and apoptosis [31]. Our results indicate that PEA3 and c-Jun strongly transactivate the distal region -3135/-2307 of the MUC4 promoter in CAPAN-1 and -2 cells and the proximal region -219/-1 in CAPAN-2 cells. The activation of the promoter was associated with an increase in endogenous MUC4 expression in these cells, especially in CAPAN-1 cells. Moreover, the fact that the amounts of c-Jun engaged in active transcriptional complexes are higher in CAPAN-1 and -2 when compared with that in PANC-1 cells further document the key regulatory role of this factor in MUC4 expression. The underlying mechanisms resulting in different levels of c-Jun activity are currently unknown. Differences in the activity of JNK (c-Jun N-terminal kinase) pathway, responsible for the activation of c-Jun by phosphorylation [31], seem unlikely since the MEKK1–JNK pathway is active in PANC-1 cells [32]. An involvement of the extracellular-signal-regulated kinase pathway that is known to play an important role in PANC-1 proliferation [33] could not be currently ruled out. Interestingly, both PEA3 and its cofactor c-Jun transcriptional activities are regulated by the MAPK pathway. JNK can induce AP-1 activity by phosphorylation of c-Jun [31]. Similarly, PEA3 activity is positively regulated by the extracellular-signal-regulated kinase and JNK pathways [29]. Further studies are required to determine the nature of the signalling pathway(s) that regulate PEA3 activity in pancreatic cancer cells and whether PEA3 is a downstream target gene of ErbB-2.

Our results demonstrate that PEA3 transactivates the *MUC4* promoter in co-operation with c-Jun in WD pancreatic tumour cells. These results are consistent with previous data showing that c-Jun synergizes with PEA3 to transactivate the matrilysin promoter in kidney epithelial cells [27]. Moreover, the activity of several promoters including those of metalloproteinase-13 [25] and cyclooxygenase-2 (*COX-2*) [34] requires closely spaced *cis*-elements for PEA3 and AP-1, suggesting that these transcription factors function synergistically. PEA3 alone induces the expression of several metalloproteinase genes such as collagenase-IV by MCF-7 cells, thus promoting the degradation of the extracellular matrix [35]. Therefore the Ets transcription factors and especially PEA3 are involved in the invasiveness of



Scheme 1 Model for the roles of PEA3 both on the regulation of *MUC4* and *ErbB-2* expression in relationship with the proliferation–differentiation balance in pancreatic cancer cells

+, stimulating effect; -, inhibiting effect; ?, not determined.

tumour cells [36]. However, the function of PEA3 may not be limited to this step of tumorigenesis, since it also regulates the expression of other genes such as *COX-2* [34], *ErbB-2* [16,17] and *MUC4* (the present study), which are known to modulate cell proliferation and/or apoptosis [37,38]. Our working hypothesis was that PEA3 may induce the expression of both *MUC4* and *ErbB-2* simultaneously thereby promoting the formation of a stable complex between these two proteins at the membrane level and consequently the phosphorylation of ErbB-2, followed by the activation of intracellular signalling pathways. These pathways are either linked to epithelial cell proliferation and cell transformation [38] or to epithelial cell differentiation, depending on the presence of neuregulin, an ErbB-3 ligand [39].

In normal epithelial cells, rat Muc4 is apically localized [39,40], whereas the ErbB-2 tyrosine kinase receptor is present at the basolateral membrane together with the other ErbB receptors. This localization depends on a specific interaction between the C-terminal end of ErbB-2 and the PDZ domain of ERBIN [41], and occurs only when ErbB-2 is not phosphorylated. Recently, Ramsauer et al. [40] demonstrated that rat Muc4 was capable of triggering ErbB-2 translocation from the basolateral to the apical membrane of polarized human colon carcinoma Caco-2 cells. This relocalization prevents the receptor interacting with partners (other ErbB receptors) to form an array of homo- and heterodimers and promotes the differentiated state of the cell. Interestingly, our results indicate that PEA3 could act both as an activator of MUC4 expression and as a repressor of ErbB-2 expression in WD pancreatic cancer cells. Such a repression of ErbB-2 expression by PEA3 has already been observed in breast and ovarian cancer cell lines [17]. This inhibitory function seems to be restricted to PEA3 since ESX (epithelial restricted with serine box)/Elf-3, an epithelial-specific Ets protein, transactivates the ErbB-2 promoter in mammary cells [42], and is capable of conferring aggressive and metastatic properties to the non-tumorigenic human mammary epithelial MCF-12A cells [43]. Moreover, Brembeck et al. [44] have previously demonstrated that ESX suppresses the promoter activity of keratin 4, which plays a key role in the early differentiation of the oesophageal squamous epithelium. These results

suggest a link between up-regulation of ErbB-2, promotion of cell transformation and inhibition of differentiation. In contrast, the stable expression of PEA3, which represses *ErbB-2* promoter activity in breast cancer cells [17], did not induce a cell transformation in MCF-12A cells [43]. Therefore several lines of evidence suggest that PEA3 down-regulates *ErbB-2* oncogene expression in tumour cells. The fact that concomitantly, PEA3 up-regulates the expression of *MUC4*, an epithelial marker merely expressed in differentiated cells [39,40] suggests that PEA3 promotes the differentiation of pancreatic cancer cells. Our results thus favour the hypothesis that PEA3 could be one of the key regulators of the differentiation/proliferation balance in pancreatic cancer cells (Scheme 1).

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